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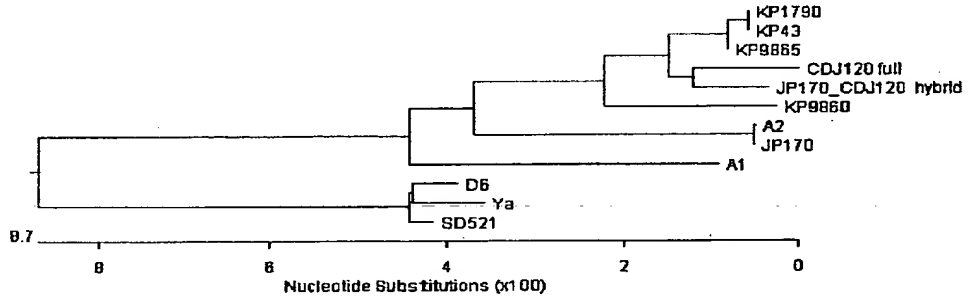
(54) Title: SUBTILASES



Phylogenetic tree

(57) Abstract: The present invention relates to novel JP170 like subtilases from wild-type bacteria, hybrids thereof and to methods of construction and production of these proteases. Further, the present invention relates to use of the claimed subtilases in detergents, such as a laundry or an automatic dishwashing detergent.

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SUBTILASES

SEQUENCE LISTING AND DEPOSITED MICROORGANISMS

Sequence listing

The present invention comprises a sequence listing.

Deposit of biological material

The following biological material has been deposited under the terms of the Budapest Treaty with the Deutsche Sammlung von Mikroorganismen und Zellkulturen and given the following accession number:

Deposit	Accession Number	Date of Deposit
JP170/CDJ120 hybrid	DSM16711	15 September 2004
CDJ120 mature	DSM16721	15 September 2004

The deposit DSM16711 contain a plasmid comprising a fragment of DNA encoding the open reading frame of the hybrid subtilase gene (JP170/CDJ120 hybrid), whereas the deposit DSM16721 contain a plasmid comprising a fragment of DNA encoding the mature segment the subtilase gene (CDJ120).

FIELD OF THE INVENTION

The present invention relates to novel JP170 like subtilases from wild-type bacteria, hybrids thereof and to methods of construction and production of these proteases. Further, the present invention relates to use of the claimed subtilases in detergents, such as a laundry detergent or an automatic dishwashing detergent.

BACKGROUND OF THE INVENTION

Enzymes have been used within the detergent industry as part of washing formulations for more than 30 years. Proteases are from a commercial perspective the most relevant enzyme in such formulations, but other enzymes including lipases, amylases, cellulases, hemicellulases or mixtures of enzymes are also often used.

The search for proteases with appropriate properties include both discovery of naturally occurring proteases, i.e. so called wild-type proteases but also alteration of well-known proteases by e.g. genetic manipulation of the nucleic acid sequence encoding said proteases. One family of proteases, which is often used in detergents, is the subtilases. This family has

been further grouped into 6 different sub-groups (Siezen F... ... ., . Protein Science, 6, 501-523). One of these sub-groups, the Subtilisin family was further divided into the subgroups of "true subtilisins (I-S1)", "high alkaline proteases (I-S2)" and "intracellular proteases". Siezen and Leunissen identified also some proteases of the subtilisin family, but not belonging to any of the subgroups. The true subtilisins include proteases such as subtilisin BPN' (BASBPN), subtilisin Carlsberg (ALCALASE®, NOVOZYMES A/S) (BLSCAR), mesentericopeptidase (BMSAMP) and subtilisin DY (BSSDY). The high alkaline proteases include proteases such as subtilisin 309 (SAVINASE®, NOVOZYMES A/S) (BLSAVI) subtilisin PB92 (BAALKP), subtilisin BL or BLAP (BLSUBL), subtilisin 147 (ESPERASE®, NOVOZYMES A/S), subtilisin Sendai (BSAPRS) and alkaline elastase YaB. Outside this grouping of the subtilisin family a further subtilisin subgroup was recently identified on the basis of the 3-D structure of its members, the TY145 like subtilisins. The TY145 like subtilisins include proteases such as TY145 (a subtilase from Bacillus sp. TY145, NCIMB 40339 described in WO 92/17577) (BSTY145), subtilisin TA41 (BSTA41), and subtilisin TA39 (BSTA39).

The JP170 subtilase type was first described as protease A in WO 88/01293 to Novozymes A/S disclosing four strains producing this type of protease. Later US patent 5,891,701 to Novozymes Biotech disclosed the amino acid sequence of JP170 and the DNA sequence encoding it. The patents JP7-62152 and JP 4197182 to Lion Corp. disclosed the alkaline protease Yb produced by Bacillus sp. Y that is homologous to JP170 and the DNA sequence encoding Yb. *Bacillus* sp. Y also produces the protease Ya (Geneseq P entry AAR26274). And in addition US 6,376,227 to Kao Corp. discloses physical characteristics as well as DNA and polypeptide sequences of alkaline proteases KP43, KP1790 and KP9860 which are also homologous to JP170. Recently genetic engineered variants of the KP43, KP9860 and Ya proteases among others were disclosed in EP 1 209 233, which also disclosed protease A-2 from Bacillus sp. NCIB12513. Kao Corp. also disclosed the proteases KSM-KP9865 and A-1 in US 2004/072321. Other known proteases belonging to this group are Protease E-1 derived from *Bacillus* sp. strain No. D6 (FERM P-1592), JP7407101, Protease SD521 derived from *Bacillus* sp. strain SD-521 (FERM BP-11162), JP9108211, and protease A1 derived from NCIB12289, WO 88/01293 to Novozymes A/S.

BRIEF DESCRIPTION OF THE INVENTION

The inventors have isolated novel proteases belonging to the JP170 like proteases subgroup of the subtilisin family that possess advantageous properties, such as improved detergent stability.

Furthermore the inventors have inserted truncated forms of the genes encoding various members of this subgroup into the gene encoding the JP170 protease thereby creating hybrid

JP170 like proteases exhibiting improved performance in cor...

The invention therefore in a further embodiment provides hybrid proteases.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1, Phylogenetic tree showing the relationship of the mature subtilase peptide sequences were constructed upon alignment with default settings in the ClustalW function of program MegAlignTM version 5.05 in DNAStar™ program package.

Figure 2, Matrix with amino acid sequence identities of the enzymes of the invention and the closest prior art known to the applicant.

DEFINITIONS

Prior to discussing this invention in further detail, the following terms and conventions will first be defined.

For a detailed description of the nomenclature of amino acids and nucleic acids, we refer to WO 00/71691 page 5, hereby incorporated by reference. A description of the nomenclature of modifications introduced in a polypeptide by genetic manipulation can be found in WO 00/71691 page 7-12, hereby incorporated by reference.

The term "subtilases" refer to a sub-group of serine proteases according to Siezen *et al.*, *Protein Engng.* 4 (1991) 719-737 and Siezen et al. *Protein Science* 6 (1997) 501-523. Serine proteases or serine peptidases is a subgroup of proteases characterised by having a serine in the active site, which forms a covalent adduct with the substrate. Further the subtilases (and the serine proteases) are characterised by having two active site amino acid residues apart from the serine, namely a histidine and an aspartic acid residue.

The subtilases may be divided into 6 sub-divisions, i.e. the Subtilisin family, the Thermitase family, the Proteinase K family, the Lantibiotic peptidase family, the Kexin family and the Pyrolysin family.

The Subtilisin family (EC 3.4.21.62) may be further divided into 3 sub-groups, i.e. I-S1 ("true" subtilisins), I-S2 (highly alkaline proteases) and intracellular subtilisins. Definitions or grouping of enzymes may vary or change, however, in the context of the present invention the above division of subtilases into sub-division or sub-groups shall be understood as those described by Siezen et al., *Protein Engng.* 4 (1991) 719-737 and Siezen et al. *Protein Science* 6 (1997) 501-523.

The term "parent" is in the context of the present invention to be understood as a protein, which is modified to create a protein variant. The parent protein may be a naturally occurring (wild-type) polypeptide or it may be a variant thereof prepared by any suitable means. For instance, the parent protein may be a variant of a naturally occurring protein which has been modified by substitution, chemical modification, deletion or truncation of one or more

amino acid residues, or by addition or insertion of one o. ... amino acid sequence, of a naturally-occurring polypeptide. Thus the term "parent subtilase" refers to a subtilase which is modified to create a subtilase variant.

The term "hybrid" is in the context of this invention to be understood as a protein that has been modified by replacing one or more segments of the gene encoding the parent protein with corresponding segments derived from genes encoding another protein.

The term "core" in the context of this invention is to be understood as a segment that comprises a substantial part of the subtilase gene including the part encoding the active site and a substantial part of the rest of the subtilase molecule, to provide unique traits to a hybrid.

The term "modification(s)" or "modified" is in the context of the present invention to be understood as to include chemical modification of a protein as well as genetic manipulation of the DNA encoding a protein. The modification(s) may be replacement(s) of the amino acid side chain(s), substitution(s), deletion(s) and/or insertions in or at the amino acid(s) of interest. Thus the term "modified protein", e.g. "modified subtilase", is to be understood as a protein which contains modification(s) compared to a parent protein, e.g. subtilase.

"Homology" or "homologous to" is in the context of the present invention to be understood in its conventional meaning and the "homology" between two amino acid sequences should be determined by use of the "Similarity" defined by the GAP program from the University of Wisconsin Genetics Computer Group (UWGCG) package using default settings for alignment parameters, comparison matrix, gap and gap extension penalties. Default values for GAP penalties, i.e. GAP creation penalty of 3.0 and GAP extension penalty of 0.1 (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711). The method is also described in S.B. Needleman and C.D. Wunsch, Journal of Molecular Biology, 48, 443-445 (1970). Identities can be extracted from the same calculation. The homology between two amino acid sequences can also be determined by "identity" or "similarity" using the GAP routine of the UWGCG package version 9.1 with default setting for alignment parameters, comparison matrix, gap and gap extension penalties can also be applied using the following parameters: gap creation penalty = 8 and gap extension penalty = 8 and all other parameters kept at their default values. The output from the routine is besides the amino acid alignment the calculation of the "Percent Identity" and the "Similarity" between the two sequences. The numbers calculated using UWGCG package version 9.1 is slightly different from the version 8.

The term "position" is in the context of the present invention to be understood as the number of an amino acid in a peptide or polypeptide when counting from the N-terminal end of said peptide/polypeptide. The position numbers used in the present invention refer to different subtilases depending on which subgroup the subtilase belongs to.

DETAILED DESCRIPTION OF THE INVENTION

Construction of degenerated primers

Degenerated primers were constructed from an alignment of genes of already known proteases such as Ya, KAO KSM-43 and JP170. The primers were degenerated in order to allow screening for protease gene fragments different from Ya, KAO KSM-43 and JP170.

PCR screening

From the company culture collection a selection of bacterial strains were included in a PCR screening using the primers SF16A767F and SF16A1802R. The expected size of the PCR product was 1050 nucleotides. All PCR products of the expected size were sequenced in two sequence reaction using one of each of the same two primers. The nucleotide sequences were translated to amino acid sequences, and the diversity analysed by comparative peptide sequence analysis.

Based on the results of the screening a number of enzymes were selected for further investigation. The selected enzymes are shown in Figure 1, and they both represent new enzyme molecules and representatives of the prior art. The enzyme selected for further investigation is CDJ120 which can be seen as forming a separate group in Figure 1. Also hybrid subtilases produced as described below can be seen in Figure 1.

Based on these results the inventors decided to move on with a dual approach; expression of the PCR product by in frame fusions to N and C terminal parts of the known protease of *Bacillus halmapalus* strain JP170 and inverse PCR to get the full sequences of selected enzymes.

Expression of hybrid proteases

Description of SOE PCR

By SOE PCR (SOE: Splicing by Overlapping Extension) hybrid gene products comprising 5 segments were generated as described in Example 2. The hybrid subtilase genes are used for production of a mature protease enzyme of about 433 amino-acids and a molecular weight of approximately 45 kd. The first segment is the nucleotide sequence encoding the pro sequence of JP170 protease (that is not a part of the mature protease) and 40 amino acids of the N terminal of the mature JP170 protease. This is followed by a fusion primer segment encoding 8 amino acids (this segment may contain sequence variation due to the degeneration of the primer SF16A767F). The third segment is encoding the approximately 343 amino acid long core. This segment includes the sequence encoding the active site of the protease. This is followed by a fusion primer segment encoding 7 amino acids (this segment

may contain variation due to the degeneration of the prime. [illegible] is encoding the 35 amino acids of the C terminal of the JP170 protease.

SOE PCR products based on core segments from the strains CDJ120 (SEQ ID NO:3) (the SEQ ID NO of the gene sequence encoding the mature hybrid protease is indicated in brackets) were generated.

The core of the subtilase of the invention may comprise 50-420 amino acid residues, preferably 50-100 amino acid residues, 100-150 amino acid residues, 150-200 amino acid residues 200-250 amino acid residues, 250-300 amino acid residues, 300-350 amino acid residues, 350-400 amino acid residues, 400-420 amino acid residues. Especially preferred is a core segment comprising approximately 343 amino acid residues.

The N terminal end of the core segment is located in one of positions 1-10, 10-20, 20-30, 30-40, 40-50, 50-60 or 60-70 of the subtilase of SEQ ID NO:4. The C terminal end of the core segment is located in one of positions 70-80, 80-90, 90-100, 100-150, 150-200, 200-250, 250-300, 300-320, 320-340, 340-360, 360-380, 380-400, 400-420 of the subtilase of SEQ ID NO:4. In a preferred embodiment the core of the subtilase of the invention comprises the amino acids in position 49-392 of the hybrid JP170/CDJ120 (SEQ ID NO:4).

The core sequence preferably has 99.2% identity with the amino acids in position 49-392 of SEQ ID NO:4. More preferably the core sequence has 99.3% identity, 99.5% identity, 99.7% identity or 99.9% identity with SEQ ID NO:4.

The corresponding nucleotides encoding the core segment can be seen in SEQ ID NO:3. In a preferred embodiment the core of the subtilase of the invention is encoded by the nucleotides in position 145-1177 of the hybrid JP170/CDJ120 (SEQ ID NO:3).

The N and C terminals of the hybrids of the present invention could equally well be selected from other subtilases, such as BLSCAR, BMSAMP, BASBPN or BSSDY of I-S1, BLSAVI, BAALKP, BLSUBL or subtilisin 147 of I-S2, a members of the TY145 like subtilases, or another member of the JP170 like subtilases.

The lengths of the N and C terminal sequences vary from 1 to approximately 150 amino acid residues. Preferably the length of the terminals are 1-20 amino acid residues, 20-40 amino acid residues, 40-60 amino acid residues, 60-80 amino acid residues, 80-100 amino acid residues, 100-120 amino acid residues, 120-150 amino acid residues.

The subtilase hybrids of the invention are preferable produced by use of the fusion primers described in Example 2, but other suitable primers may equally well be used.

Cloning of the hybrid protease

The PCR fragment was cloned into plasmid pDG268NeoMCS-PramyQ/PrcryIII/cryIIIAstab/Sav (United States Patent: 5,955,310) and transformed in *Bacillus*

subtilis. Protease positive colonies were selected and the coding sequence of the expressed enzyme from the expression construct was confirmed by DNA sequence analysis.

Cloning and expression of full length subtilase of the invention

Inverse PCR

Inverse PCR was performed with specific DNA primers designed to complement the DNA sequence of the core PCR product and chromosomal DNA extracted from the appropriate bacterial strain. Inverse PCR was made on the strains CDJ120. The inverse PCR products were nucleotide sequenced to obtain the region encoding the N and C terminal parts of the mature subtilase gene.

Production of full length subtilase

The subtilase genes were amplified with specific primers with restriction sites in the 5' end of primers that allow gene fusion with the Savinase signal peptide of plasmid pDG268NeoMCS-PramyQ/PrcryIII/cryIIIAstab/Sav (United States Patent: 5,955,310). Protease positive colonies were selected and the coding sequence of the expressed enzyme from the expression construct was confirmed by DNA sequence analysis.

Subtilases of the invention

The subtilase of the present invention include the members of the novel subgroup of Figure 1: CDJ120. According to the identity matrix of Figure 2 the sequence identity of the closest related prior art subtilase is 98.2%.

Thus, the subtilase of the present invention is at least 98.5% identical with SEQ ID NO:2 or SEQ ID NO:4. In particular said subtilase may be at least 99% or at least 99.5% identical with SEQ ID NO:2 or SEQ ID NO:4.

The subtilase of the present invention is encoded by an isolated nucleic acid sequence, which nucleic acid sequence has at least 91% identity with SEQ ID NO:1 or SEQ ID NO:3. Preferably, said nucleic acid sequence has at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity with the nucleic acid sequence shown in SEQ ID NO:1 or SEQ ID NO:3.

Further the isolated nucleic acid sequence encoding a subtilase of the invention hybridizes with a complementary strand of the nucleic acid sequence shown in SEQ ID NO:1 or SEQ ID NO:3 preferably under low stringency conditions, at least under medium stringency conditions, at least under medium/high stringency conditions, at least under high stringency conditions, at least under very high stringency conditions.

Hybridization

Suitable experimental conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (Sodium chloride/Sodium citrate, Sambrook et al. 1989) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a concentration of 10ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. For various stringency conditions the filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS and at least 55°C (low stringency), more preferably at least 60°C (medium stringency), still more preferably at least 65°C (medium/high stringency), even more preferably at least 70°C (high stringency), and even more preferably at least 75°C (very high stringency).

VARIANTS

Combined modifications

The present invention also encompasses any of the above mentioned subtilase variants in combination with any other modification to the amino acid sequence thereof. Especially combinations with other modifications known in the art to provide improved properties to the enzyme are envisaged.

Such combinations comprise the positions: 222 (improves oxidation stability), 218 (improves thermal stability), substitutions in the Ca^{2+}-binding sites stabilizing the enzyme, *e.g.* position 76, and many other apparent from the prior art.

In further embodiments a subtilase variant described herein may advantageously be combined with one or more modification(s) in any of the positions:

27, 36, 56, 76, 87, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 120, 123, 159, 167, 170, 206, 218, 222, 224, 232, 235, 236, 245, 248, 252 and 274 (BPN' numbering).

Specifically, the following BLSAVI, BLSUBL, BSKSMK, and BAALKP modifications are considered appropriate for combination:

K27R, *36D, S56P, N76D, S87N, G97N, S101G, S103A, V104A, V104I, V104N, V104Y, H120D, N123S, G159D, Y167, R170, Q206E, N218S, M222S, M222A, T224S, A232V, K235L, Q236H, Q245R, N248D, N252K and T274A.

Furthermore variants comprising any of the modifications S101G+V104N, S87N+S101G+V104N, K27R+V104Y+N123S+T274A, N76D+S103A+V104I or N76D+V104A, or other combinations of the modifications K27R, N76D, S101G, S103A, V104N, V104Y, V104I, V104A, N123S, G159D, A232V, Q236H, Q245R, N248D, N252K, T274A in combination with any one or more of the modification(s) mentioned above exhibit improved properties.

A particular interesting variant is a variant, which, in addition to modifications according to the invention, contains the following substitutions:

S101G+S103A+V104I+G159D+A232V+Q236H+Q245R+N248D+N252K.

Moreover, subtilase variants of the main aspect(s) of the invention are preferably combined with one or more modification(s) in any of the positions 129, 131 and 194, preferably as 129K, 131H and 194P modifications, and most preferably as P129K, P131H and A194P modifications. Any of those modification(s) are expected to provide a higher expression level of the subtilase variant in the production thereof.

Methods for expression and isolation of proteins

To express an enzyme of the present invention the above mentioned host cells transformed or transfected with a vector comprising a nucleic acid sequence encoding an enzyme of the present invention are typically cultured in a suitable nutrient medium under conditions permitting the production of the desired molecules, after which these are recovered from the cells, or the culture broth.

The medium used to culture the host cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The media may be prepared using procedures known in the art (see, e.g., references for bacteria and yeast; Bennett, J.W. and LaSure, L., editors, More Gene Manipulations in Fungi, Academic Press, CA, 1991).

If the enzymes of the present invention are secreted into the nutrient medium, they may be recovered directly from the medium. If they are not secreted, they may be recovered from cell lysates. The enzymes of the present invention may be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gelfiltration chromatography, affinity chromatography, or the like, dependent on the enzyme in question.

The enzymes of the invention may be detected using specific for these proteins. These detection methods include use of specific antibodies, formation of a product, or disappearance of a substrate. For example, an enzyme assay may be used to determine the activity of the molecule. Procedures for determining various kinds of activity are known in the art.

The enzymes of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing (IEF), differential solubility (e.g., ammonium sulfate precipitation), or extraction (see, e.g., Protein Purification, J-C Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

When an expression vector comprising a DNA sequence encoding an enzyme of the present invention is transformed/transfected into a heterologous host cell it is possible to enable heterologous recombinant production of the enzyme. An advantage of using a heterologous host cell is that it is possible to make a highly purified enzyme composition, characterized in being free from homologous impurities, which are often present when a protein or peptide is expressed in a homologous host cell. In this context homologous impurities mean any impurity (e.g. other polypeptides than the enzyme of the invention) which originates from the homologous cell where the enzyme of the invention is originally obtained from.

DETERGENT APPLICATIONS

The enzyme of the invention may be added to and thus become a component of a detergent composition.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations, especially for automatic dish washing (ADW).

In a specific aspect, the invention provides a detergent additive comprising the enzyme of the invention. The detergent additive as well as the detergent composition may comprise one or more other enzymes such as a protease, a lipase, a cutinase, an amylase, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, e.g., a laccase, and/or a peroxidase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (i.e. pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The protease may be a serine protease or a metallo protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274.

Preferred commercially available protease enzymes include Alcalase™, Savinase™, Primase™, Duralase™, Esperase™, and Kannase™ (Novozymes A/S), Maxatase™, Maxacal™, Maxapem™, Properase™, Purafect™, Purafect OxP™, FN2™, and FN3™ (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g. from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, e.g. from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas sp.* strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g. from *B. subtilis* (Dartois et al. (1993), Biochemica et Biophysica Acta, 1131, 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422).

Other examples are lipase variants such as those described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.
Preferred commercially available lipase enzymes include Lipolase™ and Lipolase Ultra™ (Novozymes A/S).

Amylases: Suitable amylases (α and/or β) include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example, α-amylases obtained from *Bacillus*, e.g. a special strain of *B. licheniformis*, described in more detail in GB 1,296,839.

Examples of useful amylases are the variants d
94/18314, WO 96/23873, and WO 97/43424, especially the variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and 444.

Commercially available amylases are Duramyl™, Termamyl™, Fungamyl™ and BAN™ (Novozymes A/S), Rapidase™ and Purastar™ (from Genencor International Inc.).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus, Pseudomonas, Humicola, Fusarium, Thielavia, Acremonium,* e.g. the fungal cellulases produced from *Humicola insolens, Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757 and WO 89/09259.

Especially suitable cellulases are the alkaline or neutral cellulases having colour care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

Commercially available cellulases include Celluzyme™, Renozyme® and Carezyme™ (Novozymes A/S), Clazinase™, and Puradax HA™ (Genencor International Inc.), and KAC-500(B)™ (Kao Corporation).

Peroxidases/Oxidases: Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, e.g. from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257.
Commercially available peroxidases include Guardzyme™ (Novozymes A/S).

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e. a separate additive or a combined additive, can be formulated e.g. as a granulate, a liquid, a slurry, etc. Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide

units; ethoxylated fatty alcohols in which the alcohol contain: ..... .
in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g., a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to 70 % water and 0-30 % organic solvent, or non-aqueous.

The detergent composition comprises one or more surfactants, which may be non-ionic including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants are typically present at a level of from 0.1% to 60% by weight.

When included therein the detergent will usually contain from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid or soap.

When included therein the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine ("glucamides").

The detergent may contain 0-65 % of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, carbonate, citrate, nitrilotriacetic acid, ethylenediaminetetraacetic acid, diethylenetriaminepentaacetic acid, alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinylpyrrolidone), poly (ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine or nonanoyloxybenzenesulfonate. Alternatively, the bleaching system may comprise peroxyacids of e.g. the amide, imide, or sulfone type.

The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g., a polyol such as propylene glycol or glycerol, a sugar or

sugar alcohol, lactic acid, boric acid, or a boric acid derivati

or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the composition may be formulated as described in e.g. WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, hydrotropes, tarnish inhibitors, or perfumes.

In the detergent compositions any enzyme, in particular the enzyme of the invention, may be added in an amount corresponding to 0.01-100 mg of enzyme protein per litre of wash liquor, preferably 0.05-5 mg of enzyme protein per litre of wash liquor, in particular 0.1-1 mg of enzyme protein per litre of wash liquor.

The enzyme of the invention may additionally be incorporated in the detergent formulations disclosed in WO 97/07202 which is hereby incorporated as reference.

Typical powder detergent compositions for automated dishwashing include:

1)

Nonionic surfactant	0.4 - 2.5%
Sodium metasilicate	0 - 20%
Sodium disilicate	3 - 20%
Sodium triphosphate	20 - 40%
Sodium carbonate	0 - 20%
Sodium perborate	2 - 9%
Tetraacetyl ethylene diamine (TAED)	1 - 4%
Sodium sulphate	5 - 33%
Enzymes	0.0001 - 0.1%

2)

Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 2%
Sodium disilicate	2 - 30%
Sodium carbonate	10 - 50%
Sodium phosphonate	0 - 5%
Trisodium citrate dehydrate	9 - 30%
Nitrilotrisodium acetate (NTA)	0 - 20%
Sodium perborate monohydrate	5 - 10%
Tetraacetyl ethylene diamine (TAED)	1 - 2%
Polyacrylate polymer	

(e.g. maleic acid/acrylic acid copolymer)	
Enzymes	0.0001 - 0.1%
Perfume	0.1 - 0.5%
Water	5 - 10

3)

Nonionic surfactant	0.5 - 2.0%
Sodium disilicate	25 - 40%
Sodium citrate	30 - 55%
Sodium carbonate	0 - 29%
Sodium bicarbonate	0 - 20%
Sodium perborate monohydrate	0 - 15%
Tetraacetyl ethylene diamine (TAED)	0 - 6%
Maleic acid/acrylic acid copolymer	0 - 5%
Clay	1 - 3%
Polyamino acids	0 - 20%
Sodium polyacrylate	0 - 8%
Enzymes	0.0001 - 0.1%

4)

Nonionic surfactant	1 - 2%
Zeolite MAP	15 - 42%
Sodium disilicate	30 - 34%
Sodium citrate	0 - 12%
Sodium carbonate	0 - 20%
Sodium perborate monohydrate	7 - 15%
Tetraacetyl ethylene diamine (TAED)	0 - 3%
Polymer	0 - 4%
Maleic acid/acrylic acid copolymer	0 - 5%
Organic phosphonate	0 - 4%
Clay	1 - 2%
Enzymes	0.0001 - 0.1%
Sodium sulphate	Balance

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5)

Nonionic surfactant	1 - 7%
Sodium disilicate	18 - 30%
Trisodium citrate	10 - 24%
Sodium carbonate	12 - 20%
Monopersulphate (2 $KHSO_5.KHSO_4.K_2SO_4$)	15 - 21%
Bleach stabilizer	0.1 - 2%
Maleic acid/acrylic acid copolymer	0 - 6%
Diethylene triamine pentaacetate, pentasodium salt	0 - 2.5%
Enzymes	0.0001 - 0.1%
Sodium sulphate, water	Balance

Powder and liquid dishwashing compositions with cleaning surfactant system typically include the following ingredients:

6)

Nonionic surfactant	0 - 1.5%
Octadecyl dimethylamine N-oxide dihydrate	0 - 5%
80:20 wt.C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dihydrate	0 - 4%
70:30 wt.C18/C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous	0 - 5%
C_{13}-C_{15} alkyl ethoxysulfate with an average degree of ethoxylation of 3	0 - 10%
C_{12}-C_{15} alkyl ethoxysulfate with an average degree of ethoxylation of 3	0 - 5%
C_{13}-C_{15} ethoxylated alcohol with an average degree of ethoxylation of 12	0 - 5%
A blend of C_{12}-C_{15} ethoxylated alcohols with an average degree of ethoxylation of 9	0 - 6.5%
A blend of C_{13}-C_{15} ethoxylated alcohols with an average degree of ethoxylation of 30	0 - 4%
Sodium disilicate	0 - 33%

Sodium tripolyphosphate	0 - [illegible]
Sodium citrate	0 - 28%
Citric acid	0 - 29%
Sodium carbonate	0 - 20%
Sodium perborate monohydrate	0 - 11.5%
Tetraacetyl ethylene diamine (TAED)	0 - 4%
Maleic acid/acrylic acid copolymer	0 - 7.5%
Sodium sulphate	0 - 12.5%
Enzymes	0.0001 - 0.1%

Non-aqueous liquid automatic dishwashing compositions typically include the following ingredients:

7)

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
Alkali metal silicate	3.0 - 15.0%
Alkali metal phosphate	20.0 - 40.0%
Liquid carrier selected from higher glycols, polyglycols, polyoxides, glycolethers	25.0 - 45.0%
Stabilizer (e.g. a partial ester of phosphoric acid and a C_{16}-C_{18} alkanol)	0.5 - 7.0%
Foam suppressor (e.g. silicone)	0 - 1.5%
Enzymes	0.0001 - 0.1%

8)

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
Sodium silicate	3.0 - 15.0%
Alkali metal carbonate	7.0 - 20.0%
Sodium citrate	0.0 - 1.5%
Stabilizing system (e.g. mixtures of finely divided silicone and low molecular weight dialkyl polyglycol ethers)	0.5 - 7.0%
Low molecule weight polyacrylate polymer	5.0 - 15.0%
Clay gel thickener (e.g. bentonite)	0.0 - 10.0%

Hydroxypropyl cellulose polymer	0.-
Enzymes	0.0001 - 0.1%
Liquid carrier selected from higher lycols, polyglycols, polyoxides and glycol ethers	Balance

Thixotropic liquid automatic dishwashing compositions typically include the following ingredients:

9)

C_{12}-C_{14} fatty acid	0 - 0.5%
Block co-polymer surfactant	1.5 - 15.0%
Sodium citrate	0 - 12%
Sodium tripolyphosphate	0 - 15%
Sodium carbonate	0 - 8%
Aluminium tristearate	0 - 0.1%
Sodium cumene sulphonate	0 - 1.7%
Polyacrylate thickener	1.32 - 2.5%
Sodium polyacrylate	2.4 - 6.0%
Boric acid	0 - 4.0%
Sodium formate	0 - 0.45%
Calcium formate	0 - 0.2%
Sodium n-decydiphenyl oxide disulphonate	0 - 4.0%
Monoethanol amine (MEA)	0 - 1.86%
Sodium hydroxide (50%)	1.9 - 9.3%
1,2-Propanediol	0 - 9.4%
Enzymes	0.0001 - 0.1%
Suds suppressor, dye, perfumes, water	Balance

Liquid automatic dishwashing compositions typically include the following ingredients:

10)

Alcohol ethoxylate	0 - 20%
Fatty acid ester sulphonate	0 - 30%
Sodium dodecyl sulphate	0 - 20%
Alkyl polyglycoside	0 - 21%
Oleic acid	0 - 10%
Sodium disilicate monohydrate	18 - 33%

Sodium citrate dehydrate	18
Sodium stearate	0 - 2.5%
Sodium perborate monohydrate	0 - 13%
Tetraacetyl ethylene diamine (TAED)	0 - 8%
Maleic acid/acrylic acid copolymer	4 - 8%
Enzymes	0.0001 - 0.1%

Liquid automatic dishwashing compositions containing protected bleach particles typically include the following ingredients:

11)

Sodium silicate	5 - 10%
Tetrapotassium pyrophosphate	15 - 25%
Sodium triphosphate	0 - 2%
Potassium carbonate	4 - 8%
Protected bleach particles, e.g. chlorine	5 - 10%
Polymeric thickener	0.7 - 1.5%
Potassium hydroxide	0 - 2%
Enzymes	0.0001 - 0.1%
Water	Balance

12) Automatic dishwashing compositions as described in 1), 2), 3), 4), 6) and 10), wherein perborate is replaced by percarbonate.

13) Automatic dishwashing compositions as described in 1) - 6) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

MATERIALS AND METHODS

Method for producing a subtilase variant

The present invention provides a method of producing an isolated enzyme according to the invention, wherein a suitable host cell, which has been transformed with a DNA sequence encoding the enzyme, is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

When an expression vector comprising a DNA sequence en... into a heterologous host cell it is possible to enable heterologous recombinant production of the enzyme of the invention. Thereby it is possible to make a highly purified subtilase composition, characterized in being free from homologous impurities.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed subtilase may conveniently be secreted into the culture medium and may be recovered there-from by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulfate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Example 1

PCR screening

The core part of protease gene was amplified in a PCR reaction that included 50U/ml of Ampli-taq™ DNA polymerase (Perkin Elmer) 10x Amplitaq buffer (final concentration of $MgCl_2$ is 1.5 mM) 0.2 mM of each of the dNTPs (dATP, dCTP, dTTP and dGTP) 0.2 pmol/µl of the primers SF16A767F (CNATGCATGAAGCNTTCCGCGG, SEQ ID NO:5) ("N" is degeneration introduced by insertion of inosine)) and SF16A1802R (CNACGTTGTTNCNGCCATCCC, SEQ ID NO:6) and 1 µl template DNA. Template DNA was recovered from the various Bacillus strains using HighPure™ PCR template preparation kit (Boehringer Mannheim art. 1796828) as recommended by the manufacturer for DNA recovery from bacteria. The quality of the isolated template was evaluated by agarose gel electrophoresis. If a high molecular weight band was present the quality was accepted. PCR was run in the following protocol: 94°C, 2 minutes 40 cycles of [94°C for 30 seconds, 52°C for 30 seconds, 68°C for 1 minute] completed with 68°C for 10 minutes. PCR products were analysed on a 1% agarose gel in TAE buffer stained with Ethidium bromide to confirm a single band of app. 1050 nucleotides. The PCR product was recovered by using Qiagen™ PCR purification kit as recommended by the manufacturer. The nucleotide sequences were determined by sequencing on an ABI PRISM™ DNA sequencer (Perkin Elmer). A PCR product of CDJ120 was determined. The nucleotide sequences were translated to amino acid sequences, and the diversity analysed by comparative peptide sequence analysis. As can be seen in Figure 1 the diversity by far exceeded that of the prior art.

EXAMPLE 2

Production of subtilase hybrids

Expression of hybrid proteases, PCR amplification

In order to produce an active subtilase base... information of the partial sequencing of Example 1, the core PCR product was fused to the N and C terminal parts of the JP170 protease gene in a SOE PCR (SOE: Splicing by Overlapping Extension) reaction as described above. In the SOE PCR reaction a fusion of three PCR products are produced. The three PCR products are:

1) The N terminal part of JP170 protease gene. This PCR product is obtained by PCR using the primers
PEP192 5'- CCGCGGAATGCTTCATGCATCG -3' (SEQ ID NO:12) and
PEP200 5'- GTTCATCGATCTTCTACTATTGGGGCGAAC-3' (SEQ ID NO:13) and 1 µl template DNA. Template DNA was recovered from the various *Bacillus* strains using HighPure™ PCR template preparation kit (Boehringer Mannheim art. 1796828) as recommended by the manufacturer for DNA recovery from bacteria. The quality of the isolated template was evaluated by agarose gel electrophoresis. If a high molecular weight band was present the quality was accepted. PCR was run in the following protocol: 94°C, 2 minutes 40 cycles of [94°C for 30 seconds, 52°C for 30 seconds, 68°C for 1 minute] completed with 68°C for 10 minutes. PCR products were analysed on a 1% agarose gel in TAE buffer stained with Ethidium bromide to confirm a single band of app. 700 nucleotides.

2) The C terminal part of JP170 protease gene. This PCR product is obtained by PCR using the primers
PEP193 5'- GGGATGGCAGAAACAACGTGG -3' (SEQ ID NO:14) and
PEP201 5'-TTAAACGCGTTTAATGTACAATCGCTAAAGAAAAG -3' (SEQ ID NO:15) and 1 µl template DNA. Template DNA was recovered from the various *Bacillus* strains using HighPure™ PCR template preparation kit (Boehringer Mannheim art. 1796828) as recommended by the manufacturer for DNA recovery from bacteria. The quality of the isolated template was evaluated by agarose gel electrophoresis. If a high molecular weight band was present the quality was accepted. PCR was run in the following protocol: 94°C, 2 minutes 40 cycles of [94°C for 30 seconds, 52°C for 30 seconds, 68°C for 1 minute] completed with 68°C for 10 minutes. PCR products were analysed on a 1% agarose gel in TAE buffer stained with Ethidium bromide to confirm a single band of app. 370 nucleotides.

3) The core PCR product described in Example 1.

In the SOE PCR reaction the three PCR products are mixed and a fused product is amplified in a standard PCR protocol using the primers PEP200 and PEP201 and 1 µl template DNA. Template DNA is a mixture of the three PCR products described above (1-3). These PCR

products may be recovered using Qiaquick™ spin colu ... Germany). The quality of the isolated template was evaluated by agarose gel electrophoresis. PCR was run in the following protocol: 94°C, 2 minutes 40 cycles of [94°C for 30 seconds, 52°C for 30 seconds, 68°C for 1 minute] completed with 68°C for 10 minutes. PCR products were analysed on a 1% agarose gel in TAE buffer stained with Ethidium bromide to confirm a single band of app. 1850 nucleotides.

The digested and purified PCR fragment was ligated to the Cla I and Mlu I digested plasmid pDG268NeoMCS-PramyQ/PrcryIII/cryIIIAstab/Sav (United States Patent: 5,955,310). The ligation mixture was used for transformation into *E. coli* TOP10F' (Invitrogen BV, The Netherlands) and several colonies were selected for miniprep (QIAprep® spin, QIAGEN GmbH, Germany). The purified plasmids were checked for insert before transformation into a strain of *Bacillus subtilis* derived from *B. subtilis* DN 1885 with disrupted apr, npr and pel genes (Diderichsen et al (1990), J. Bacteriol., 172, 4315-4321). The disruption was performed essentially as described in "*Bacillus subtilis* and other Gram-Positive Bacteria," American Society for Microbiology, p.618, eds. A.L. Sonenshein, J.A. Hoch and Richard Losick (1993). Transformed cells were plated on 1% skim milk LB-PG agar plates, supplemented with 6 µg/ml chloramphenicol. The plated cells were incubated over night at 37°C and protease containing colonies were identified by a surrounding clearing zone. Protease positive colonies were selected and the coding sequence of the expressed enzyme from the expression construct was confirmed by DNA sequence analysis.

EXAMPLE 3

Production of full length subtilases

Inverse PCR

Three digestions of the chromosomal DNA of the strains CDJ120 were made using the restriction enzymes xho1, BamH1 and Pst1. Upon digestion the DNA was separated from the restriction enzymes using Qiaquick™ PCR purification kit (art. 28106, Qiagen, Germany). The digestions were religated and subjected to a PCR reaction using primers (PCR primers SEQ ID NO:7-8) designed to recognise the sequence of the PCR product already obtained. The following PCR protocols were applied: 94°C 2 min 30 cycles of [94°C for 15 s, 52°C for 30 s, 72°C for 2 min] 72°C 20 min. Using same PCR amount of primer polymerase and buffer as above. Alternatively a protocol with 94°C 2 min 30 cycles of [94°C for 15 s, 52°C for 30 s, 68°C for 3 min] 68°C 20 min. and replacing Amplitaq® and Amplitaq® buffer with Long-template Taq polymerase™ (Boehringer Mannheim) with the buffer supplied with the polymerease. The PCR reactions were analysed on 0.8% agarose gels stained with ethidium bromide. All PCR fragments were recovered and the nucleotide sequence was determined by using specific oligo primers different from those used in the PCR reaction (Sequencing primers SEQ ID

NO:9-11). In some cases the first primer did not give ..... information to characterise the entire open reading frame of the protease gene. In these cases new primers were applied either by using the sequence information obtained with the initial inverse PCR sequencing primer, or by going back to the initial PCR fragment and defining a new primer sequence.

The following primers were used for obtaining the inverse PCR and sequencing:

PCR primers

CDJ120 PCR Forward: CCGAACGGAAACCAAGGATGGG (SEQ ID NO:7)

CDJ120 PCR Reverse: GGAGCCGTTTCCTAATACAGAG (SEQ ID NO:8)

Sequencing primers

CDJ120 Forward Sequencing TTGGACCTTGTCATTACCGC (SEQ ID NO:9)

CDJ120 Reverse Sequencing1 AGACCTCCAAGTCCTCCACC (SEQ ID NO:10)

CDJ120 Reverse Sequencing2 CATTGCTTGCTGCGTATTGG (SEQ ID NO:11)

The gene sequence encoding the mature part of the protease gene of strain CDJ120 is shown in SEQ ID NO:1.

PCR amplification

In order to produce an active subtilase based on the nucleotide sequence information of the 1305 nucleotide gene segment encoding the full length mature CDJ120 subtilase (SEQ ID NO:1), a gene fusion was made to the DNA encoding the pro sequence of the JP170 protease by SOE PCR.

Two PCR fragments were amplified:

1) The nucleotide sequence encoding the full length mature CDJ120 subtilase was amplified with primers CDJ120 Mlu1_R TTAAACGCGTTTAGTTTACAATTGCCAACG (SEQ ID NO:16) and CDJ120 SOEF AATGANGTGGCCCGNGGNATTG (SEQ ID NO:17, N is inosine) using CDJ120 chromosomal DNA (this gene segment is deposited as plasmid DNA contained in DSM16721).

2) The pro sequence of the JP170 subtilase gene was amplified using primers JP170_CDJ120_SOE_R CAATGCCACGGGCCACGTCATT (SEQ ID NO:18). PEP200 5'- GTTCATCGATCTTCTACTATTGGGGCGAAC-3' (SEQ ID NO:13) and JP170 chromosomal DNA as template. Template DNA was recovered from the various *Bacillus* strains using HighPure™ PCR template preparation kit (Boehringer Mannheim art. 1796828) as recommended by the manufacturer for DNA recovery from bacteria. The quality of the

isolated template was evaluated by agarose gel electroph
band was present the quality was accepted.

Both PCR were run in the following protocol: 94°C, 2 minutes 40 cycles of [94°C for 30 seconds, 52°C for 30 seconds, 68°C for 1 minute] completed with 68°C for 10 minutes. PCR products were analysed on a 1% agarose gel in TAE buffer stained with Ethidium bromide to confirm a single band of app. 700 nucleotides.

In the subsequent SOE PCR reaction a fusion of two PCR products were produced. PCR was run in the following protocol: 94°C, 2 minutes 40 cycles of [94°C for 30 seconds, 52°C for 30 seconds, 68°C for 1 minute] completed with 68°C for 10 minutes. PCR products were analysed on a 1% agarose gel in TAE buffer stained with Ethidium bromide to confirm a single band of app. 1850 nucleotides.

The digested and purified PCR fragment was ligated to the Cla I and Mlu I digested plasmid pDG268NeoMCS-PramyQ/PrcryIII/cryIIIAstab/Sav (United States Patent: 5,955,310).

The ligation mixture was used for transformation into *E. coli* TOP10F' (Invitrogen BV, The Netherlands) and several colonies were selected for miniprep (QIAprep® spin, QIAGEN GmbH, Germany). The purified plasmids were checked for insert before transformation into a strain of *Bacillus subtilis* derived from *B. subtilis* DN 1885 with disrupted apr, npr and pel genes (Diderichsen et al (1990), J. Bacteriol., 172, 4315-4321). The disruption was performed essentially as described in "*Bacillus subtilis* and other Gram-Positive Bacteria," American Society for Microbiology, p.618, eds. A.L. Sonenshein, J.A. Hoch and Richard Losick (1993). Transformed cells were plated on 1% skim milk LB-PG agar plates, supplemented with 6 µg/ml chloramphenicol. The plated cells were incubated over night at 37°C and protease containing colonies were identified by a surrounding clearing zone. Protease positive colonies were selected and the coding sequence of the expressed enzyme from the expression construct was confirmed by DNA sequence analysis.

EXAMPLE 4

Purification and characterisation

Purification

This procedure relates to purification of a 2 liter scale fermentation for the production of the subtilases of the invention in a *Bacillus* host cell.

Approximately 1.6 liters of fermentation broth are centrifuged at 5000 rpm for 35 minutes in 1 liter beakers. The supernatants are adjusted to pH 6.5 using 10% acetic acid and filtered on Seitz Supra® S100 filter plates.

The filtrates are concentrated to approximately 400 ml using an Amicon® CH2A UF unit equipped with an Amicon® S1Y10 UF cartridge. The UF concentrate is centrifuged and filtered

prior to absorption at room temperature on a Bacitracin affin .
eluted from the Bacitracin column at room temperature using 25% 2-propanol and 1 M sodium chloride in a buffer solution with 0.01 dimethylglutaric acid, 0.1 M boric acid and 0.002 M calcium chloride adjusted to pH 7.

The fractions with protease activity from the Bacitracin purification step are combined and applied to a 750 ml Sephadex® G25 column (5 cm dia.) equilibrated with a buffer containing 0.01 dimethylglutaric acid, 0.2 M boric acid and 0.002 m calcium chloride adjusted to pH 6.5.

Fractions with proteolytic activity from the Sephadex® G25 column are combined and applied to a 150 ml CM Sepharose® CL 6B cation exchange column (5 cm dia.) equilibrated with a buffer containing 0.01 M dimethylglutaric acid, 0.2 M boric acid, and 0.002 M calcium chloride adjusted to pH 6.5.

The protease is eluted using a linear gradient of 0-0.1 M sodium chloride in 2 litres of the same buffer.

In a final purification step subtilase containing fractions from the CM Sepharose® column are combined and concentrated in an Amicon® ultrafiltration cell equipped with a GR81PP membrane (from the Danish Sugar Factories Inc.).

EXAMPLE 5

Stability of subtilases

The stability of the produced subtilases was evaluated in a standard Western European dishwashing tablet detergent without other enzymes than the experimentally added subtilases. The stability of the subtilases is determined as the residual proteolytic activity after incubation of the subtilase in a detergent.

The formulation of a standard Western European Tablet detergent is defined as

Component	Percentage
Non ionic surfactants	0-10%
Foam regulators	1-10%
Bleach (per-carbonate or per-borate)	5-15%
Bleach activators (e.g. TAED)	1-5%
Builders (e.g. carbonate, phosphate, tri-phosphate, Zeolite)	50-75%
Polymers	0-15%
Perfume, dye etc.	<1%
Water and fillers (e.g. sodium sulphate)	Balance

Assay for Proteolytic Activity

The proteolytic activity was determined with casein as substrate. One Casein Protease Unit (CPU) is defined as the amount of protease liberating about 1 µM of primary amino groups

(determined by comparison with a serine standard) per min incubation for about 30 minutes at about 25°C at pH 9.5.

The proteolytic activity may also be determined by measuring the specific hydrolysis of succinyl-Ala-Ala-Pro-Leu-p-nitroanilide by said protease. The substrate is initially dissolved in for example, DMSO (Dimethyl Sulfoxide) and then diluted about 50 fold in about 0.035 M borate buffer, about pH 9.45. All protease samples may be diluted about 5-10 fold by the same borate buffer. Equal volumes of the substrate solution and sample are mixed in a well of an ELISA reader plate and read at about 405 nm at 25°C. All sample activities and concentrations are normalized to the standard protease solution activity and concentration, respectively.

A typical Western European tablet detergent for automated dishwashing was dissolved (5.5 g/L) in 9°dH water at ambient temperature maximum 30 minutes prior to start of analyses. Samples of subtilases were diluted to a concentration of 2-4 CPU/ml in Britten Robinson buffer (Britten Robinson buffer is: 40 mM Phosphate, 40 mM Acetate and 40 mM Borate) pH9.5. For the analyses every sample was divided and tested under two conditions: For the control the subtilase was diluted 1:9 in Britten Robinson buffer pH9.5 to a final volume of 1 ml. This sample was analysed immediately after dilution. For the detergent stability the subtilase sample was diluted 1:9 in detergent solution (detergent concentration in the stability test is 5 g/L) these samples were incubated at 55°C for 30 minutes prior to analysis by addition of casein substrate.

The assay was started by addition of 2 volumes of casein substrate (casein substrate was 2 g of casein (Merck, Hammerstein grade) in 100 ml of Britten Robinson buffer pH 9.5, pH was re-adjusted to 9.5 when the casein is in solution). Samples are kept isothermic at 25°C for 30 minutes. The reaction was stopped by addition of 5 ml TCA solution (TCA solution is 89.46 g of Tri-chloric acid, 149.48 g of Sodium acetate-tri-hydrate and 94.5 ml of glacial acetic acid in 2.5 L of deionised water). The samples are incubated at ambient temperature for at least 20 minutes and filtered through Whatman® paper filter no. 42.

400µl of filtrate is mixed with 3 ml OPA reagent (OPA reagent is composed of: 3.812 g of borax, 0.08% EtOH, 0.2% DTT and 80 mg of o-phthal-dialdehyd in 100 ml water). Absorption at 340nM is measured and CPU is calculated from the concentration of free amines on a standard of a solution of 0.01% L-serine (Merck art. 7769).

Enzymatic proteolysis of reference proteases in the typical Western European tablet detergent:

	CPU/L		
Protease	Control	Detergent	% activity
Alcalase	250	31	13%
Esperase	220	116	53%
Savinase	538	21	4%
Everlase16L	2383	86	4%
Ovozyme	2848	44	2%

BLAP-S	36	1	3%
JP170	754	370	49%

Enzymatic proteolysis of cloned hybrid proteases of the invention in the typical Western European tablet detergent. The reference is JP170:

	CPU/l		
Hybrid	Control	Detergent	% activity
JP170	67	36	53%
JP170	66	38	57%
CDJ120-1	44	34	77%
CDJ120-1	46	38	83%

As can be seen from the results the subtilases of the invention exhibit improved stability in a detergent as compared to the prior art.

CLAIMS

1. A subtilase enzyme which

a) has an amino acid sequence which is at least 98.5% identical with the sequence of SEQ ID NO:2 or SEQ ID NO:4 or

b) is encoded by a nucleotide sequence obtainable from a deposited strain selected from the group consisting of DSM16711 and DSM16721.

2. An isolated polypeptide having subtilase activity, which polypeptide is selected from the group consisting of:

a) a polypeptide which is encoded by a nucleic acid sequence which is at least 91% identical with SEQ ID NO:1 or SEQ ID NO:3,

b) a polypeptide comprising a segment, which segment is encoded by a subsequence of the DNA sequence set forth in SEQ ID NO:1 or SEQ ID NO:3, wherein said subsequence consists of the nucleic acids starting from one of positions 1-210 and ending with one of positions 600-1260 or

c) a polypeptide which is encoded by a nucleic acid sequence which is capable of hybridizing under medium/high stringency conditions with (i) the nucleic acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:3, (ii) its complementary strand, or (iii) a subsequence of (i) or (ii).

3. A nucleic acid sequence which is contained on a plasmid in the deposited strain DSM16711 or DSM16721.

4. A nucleic acid sequence as shown in SEQ ID NO:1 or SEQ ID NO:3.

5. A nucleic acid construct comprising the nucleic acid sequence of claim 4 operably linked to one or more control sequences capable of directing the expression of the polypeptide in a suitable expression host.

6. A recombinant expression vector comprising the nucleic acid construct of claim 5, a promoter, and transcriptional and translational stop signals.

7. The vector according to claim 6, further comprising a selectable marker.

8. A recombinant host cell comprising the nucleic acid construct of claim 5.

9. The cell according to claim 8, wherein the nucleic acid c

10. The cell according to claim 9, wherein the nucleic acid construct is integrated into the host cell genome.

11. The cell according to claim 10, wherein the nucleic acid sequence encodes an amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4.

12. The cell according to claim 11, wherein the nucleic acid sequence is set forth in SEQ ID NO:1 or SEQ ID NO:3.

13. A method for producing the polypeptide of claim 1 comprising (a) cultivating a *Bacillus* strain to produce a supernatant comprising the polypeptide; and (b) recovering the polypeptide.

14. A method for producing the polypeptide of claim 1 comprising (a) cultivating a host cell comprising a nucleic acid construct comprising a nucleic acid sequence encoding the polypeptide under conditions conducive to expression of the polypeptide; and (b) recovering the polypeptide.

15. A core sequence of the subtilase of claim 1, which core sequence consists of the amino acids starting from one of positions 1-70 and ending with one of position 200-420 of SEQ ID NO:2 or SEQ ID NO:4.

16. The core sequence of claim 15, which has 96% identity with the amino acids in position 49-391 of SEQ ID NO:2 or SEQ ID NO:4.

17. Use of the core sequence of claim 15 or 16 for production of a hybrid subtilase.

18. A hybrid subtilase comprising a core sequence of claim 15 or 16.

19. Use of a subtilase of claim 1 or a hybrid subtilase of claim 18 in a detergent.

20. A detergent composition comprising a subtilase of claim 1 or a hybrid subtilase of claim 18.

21. The detergent composition of claim 20, which is a laundry detergent or an automatic dishwashing detergent.

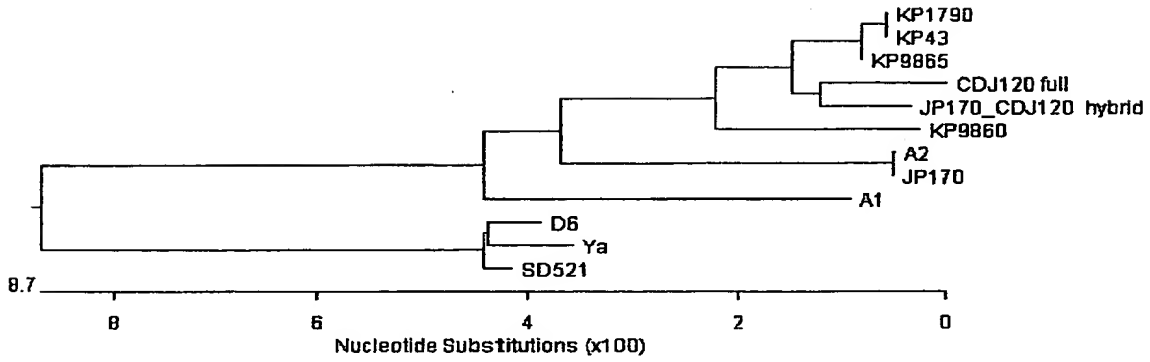

Fig. 1

Phylogenetic tree

	CDJ120	D6	JP170	KAO-A1	KSM-KP43	KSM-KP9860	KSM-KP9865	JP170/CDJ120	ProtY
D6	86,8								
JP170	92,9	88,9							
KAO-A1	92,9	88,5	91,9						
KSM-KP43	97,7	87,5	93,5	93,5					
KSM-KP9860	96,1	88,2	93,1	92,4	96,5				
KSM-KP9865	97,9	87,8	93,8	93,8	99,8	96,8			
JP170/CDJ120	97,9	88	94,7	92,9	97,9	95,9	98,2		
ProtY	86,6	98,8	88,7	88	87,5	88,2	87,8	88	
SD521	87,1	99,3	89,1	88,7	88	88,7	88,2	88,5	99,1

Fig. 2

Identity matrix

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            20                  25                  30

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        35                  40                  45

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    50                  55                  60

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atc atg gat agc ggt gga gga ctt gga ggt cta cct tcg aat ctg caa      336
Ile Met Asp Ser Gly Gly Gly Leu Gly Gly Leu Pro Ser Asn Leu Gln
            100                 105                 110

acc tta ttc agc caa gca tac agt gca ggt gcc aga att cat aca aac      384
Thr Leu Phe Ser Gln Ala Tyr Ser Ala Gly Ala Arg Ile His Thr Asn
        115                 120                 125

tcc tgg gaa gca gcg gta aat ggg gct tac acg aca gat tca agg aat      432
Ser Trp Glu Ala Ala Val Asn Gly Ala Tyr Thr Thr Asp Ser Arg Asn
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gtg gat gac tat gta cga aaa aat gat atg acg att ctt ttc gct gcc      480
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    290                 295                 300

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305                 310                 315                 320

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Val Asn Glu Ser Ser Ser Leu Ser Thr Ser Gln Lys Ala Thr Tyr Ser
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Val Lys Asn Arg Gly Ile Thr Pro Lys Pro Ser Leu Leu Lys Ala Ala
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ctt gat aca gga aga aat gac agt tcg atg cat gaa gca ttc cgc ggg      144
Leu Asp Thr Gly Arg Asn Asp Ser Ser Met His Glu Ala Phe Arg Gly
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65                  70                  75                  80

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Ile Met Asp Ser Gly Gly Gly Leu Gly Gly Leu Pro Ser Asn Leu Gln
            100                 105                 110

acc tta ttc agc caa gca tac agt gca ggt gcc aga att cat aca aac      384
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Phe Thr Ala Thr Ala Gly Lys Pro Leu Lys Ile Ser Leu Val Trp Ser
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Asp Leu Val Ile Thr Ala Pro Asn Gly Thr Gln Tyr Val Gly Asn Asp
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Asn Val Phe Ile Asn Ala Pro Gln Ser Gly Thr Tyr Thr Val Glu Val
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0-1	Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.50 (Build 0002.173)
0-2	International Application No.	PCT/DK 2005/000598
0-3	Applicant's or agent's file reference	10684.204-WO

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	1
1-2	line	7-17
1-3	Identification of deposit	
1-3-1	Name of depositary institution	DSMZ DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	15 September 2004 (15.09.2004)
1-3-4	Accession Number	DSMZ 16711
1-5	Designated States for Which Indications are Made	all designations
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	page	1
2-2	line	7-17
2-3	Identification of deposit	
2-3-1	Name of depositary institution	DSMZ DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
2-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
2-3-3	Date of deposit	15 September 2004 (15.09.2004)
2-3-4	Accession Number	DSMZ 16721
2-5	Designated States for Which Indications are Made	all designations

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	Ja
0-4-1	Authorized officer	J. Dahl

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	

Additional Indications (Form PCT/RO/134)

Statement regarding the "expert option"

Deposited microorganisms:

Deposit	Accession Number	Date of Deposit
JP170/CDJ120 hybrid	DSM16711	15 September 2004
CDJ120 mature	DSM16721	15 September 2004

With respect to the deposited microorganism set out on Form PCT/RO/134 we request the so-called expert option:

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited micro-organism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

Bagsværd, 20-SEP-2005

Novozymes A/S

.....

Rasmus R. Hansen, Patent Counsel

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2005/000598

A. CLASSIFICATION OF SUBJECT MATTER
C12N9/54 C12N15/57 C12N15/62 C11D3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, WPI Data, EMBASE, BIOSIS, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 029 920 A (KAO CORPORATION) 23 August 2000 (2000-08-23) the whole document	1-21
X	DATABASE EMBL 'Online! 2 September 2003 (2003-09-02), "Bacillus sp. KSM-9865 gene for protease, complete cds." XP002354927 retrieved from EBI accession no. EM_PRO:AB084155 Database accession no. AB084155 the whole document	2
X A	US 2004/072321 A1 (SATO TSUYOSHI ET AL) 15 April 2004 (2004-04-15) cited in the application the whole document	2 1

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 2005	02/12/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Wiame, I

3

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2005/000598

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAEKI KATSUHISA ET AL: "Novel oxidatively stable subtilisin-like serine proteases from alkaliphilic Bacillus spp.: Enzymatic properties, sequences, and evolutionary relationships" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 279, no. 2, 20 December 2000 (2000-12-20), pages 313-319, XP002238277 ISSN: 0006-291X	2
A	the whole document	1
X	WO 98/56927 A (NOVO NORDISK BIOTECH, INC) 17 December 1998 (1998-12-17) cited in the application	2
A	the whole document	1
A	WO 2004/067737 A (NOVOZYMES A/S; SVENDSEN, ALLAN; DRABORG, HENRIETTE; TINDBAEK, NIKOLAJ) 12 August 2004 (2004-08-12) abstract; examples 2,3	15,18
A	MAURER KARL-HEINZ: "Detergent proteases." CURRENT OPINION IN BIOTECHNOLOGY. AUG 2004, vol. 15, no. 4, August 2004 (2004-08), pages 330-334, XP002355089 ISSN: 0958-1669 the whole document	1-21

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2005/000598

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EP 1029920 A	23-08-2000	AU 732369 B2	26-04-2001
		AU 9457998 A	27-04-1999
		CN 1130462 C	10-12-2003
		ID 24410 A	20-07-2000
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		JP 3479509 B2	15-12-2003
		US 6376227 B1	23-04-2002
US 2004072321 A1	15-04-2004	CN 1487080 A	07-04-2004
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		EP 0988386 A2	29-03-2000
		JP 2001514529 T	11-09-2001
		US 5891701 A	06-04-1999
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